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## **Identification and quantification of biomarkers to confirm the poisoning by Ginkgo biloba seeds in a 2-year-old boy**

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## Identification and quantification of biomarkers to confirm the poisoning by *Ginkgo biloba* seeds in a 2-year-old boy

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The seeds of *Ginkgo biloba* are commonly eaten in Japan, Korea and China, but it is important to know that they can be responsible for poisoning, especially in young children. Poisoning due to *Ginkgo biloba* seeds must also be considered as a possible clinical event in European countries, where Asian cuisine has recently become popular. This paper reports the case of a 23-months-old previously healthy male child who experienced two afebrile tonic-clonic seizures after the consumption of an unknown amount of Ginkgo seeds. The poisoning was identified by searching both in blood and urine for 4'-O-methylpyridoxine (MPN) as a specific biomarker for ginkgo poisoning, using an optimized and validated HPLC method with fluorimetric detection. The MPN concentrations in serum were 16.5 and 6.2 ng mL<sup>-1</sup> 14 and 20 hours after the exposure, respectively, while the urine concentration was below the limit of detection (1.35 ng mL<sup>-1</sup>). The involvement of ginkgo seeds in the described poisoning was confirmed by searching ginkgolide terpene lactones in urine, where the ratio between excreted ginkgolide A : B : C was 1 : 40 : 2. The analytical data obtained by looking for biomarkers in serum and urine demonstrate that even low values of MPN/ginkgolide concentration can be responsible for convulsions and other adverse effects.

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## Introduction

*Ginkgo biloba* (commonly known as ginkgo or Maidenhair tree) is an ancient tree with distinctive fan-shaped leaves, belonging to the family of *Ginkgoaceae*. Leaves and seeds are the parts most usually consumed, both as food (roasted or cooked seeds) and as extracts in food supplements and traditional medicines. The leaf extract is widely used in Europe as a traditional drug to improve the symptoms of early-stage Alzheimer's disease, vascular dementia, peripheral claudication and tinnitus of vascular origin.<sup>1–4</sup> The adverse effects of *Ginkgo biloba* preparations containing leaves are numerous; approximately 30% of these cases are due to interaction with

therapeutic drugs, including acetyl salicylic acid, ibuprofen and warfarin.<sup>5</sup>

The haemorrhagic complications, due to poisoning or interaction with prescription drugs, are probably due to the antiplatelet activity of ginkgolides, and in particular of Ginkgolide B.<sup>6</sup>

*Ginkgo biloba* seeds are commonly eaten in Japan, Korea and China for their nutritive value, but it is important to know that they can be responsible for poisoning, especially in young children.<sup>7,8</sup> Even though they are infrequent, clinical cases of ginkgo seed poisoning show severe symptoms, including tonic or clonic convulsion, vomiting, irritability and loss of consciousness.<sup>7–9</sup>

Poisoning by ginkgo seeds is due to the neurotoxic compound 4'-O-methylpyridoxine (MPN), which is chemically related to vitamin B<sub>6</sub> and interfere with its biosynthesis, metabolism and function. The chemical structures of MPN and vitamin B<sub>6</sub> are reported in Fig. 1.

*Ginkgo biloba* seeds contain MPN at concentrations ranging between 170 and 400 µg g<sup>-1</sup> of raw seeds.<sup>10–13</sup> The toxin is relatively heat-resistant so that cooking or other treatments only partially inactivate it.<sup>14–16</sup>

Kajiya & Fujii (2002)<sup>7</sup> described a case of poisoning in a 2-year-old girl who showed unexplained convulsions with vomiting, diarrhoea and irritability. The event was associated

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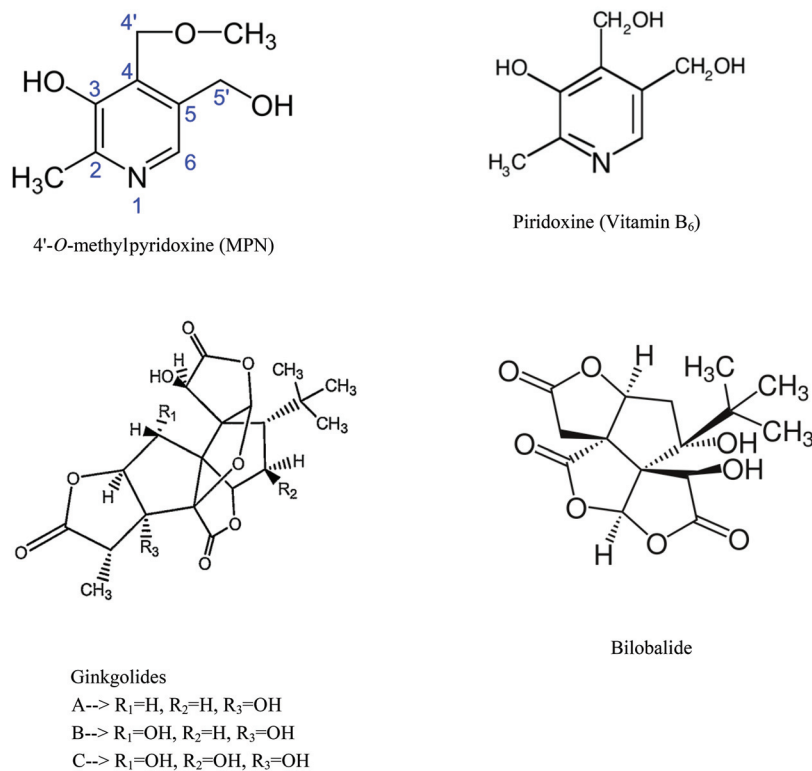


Fig. 1 Chemical structures of 4'-O-methylpyridoxine, pyridoxine (vitamin B<sub>6</sub>), ginkgolides and bilobalide.

with the ingestion of 50 to 60 roasted ginkgo seeds, as reported by the parents. Similarly, a case of poisoning was described in another 2-year-old girl who had eaten about 50 ginkgo seeds 4 hours before the onset of symptoms<sup>8</sup> and a case of poisoning with ginkgo nuts was observed in a healthy 36-year-old woman, who consumed 70–80 seeds cooked in a microwave oven.<sup>9</sup> The pathophysiologic mechanisms responsible for convulsions related to ginkgo poisoning are not fully understood. As known from *in vivo* studies, 4'-O-methylpyridoxine (MPN) competes with vitamin B<sub>6</sub>, which is a cofactor of glutamate decarboxylase; as a consequence, MPN indirectly inhibits the activity of this enzyme resulting in a decrease of the  $\gamma$ -aminobutyric acid (GABA) level in brain, which predisposes to convulsions.<sup>9,17,18</sup>

The study reported in this paper describes a case of ginkgo poisoning which occurred in Switzerland in a 23-months-old boy, where the association with the ingestion of ginkgo seeds was confirmed by measuring biomarkers both in blood and urine.

## Materials

### Clinical data

A 23-months-old previously healthy male child of Asian origin experienced two afebrile tonic-clonic seizures for 15–30 seconds. He had a 2 hour period of vomiting and somnolence. The parents reported that the child ingested an unknown

amount of Ginkgo seeds 10 hours before the beginning of symptoms. The subsequent clinical course was uneventful with full recover. Blood samples were taken 14 and 20 hours after the ingestion of seeds, while urine was collected only 14 hours after intake. No ethics committee/institutional review board approval was necessary for the analyses performed in this case, since they were requested to identify poisoning source. Informed consent to the publication of this case was obtained from the patient's family.

### Purified standards

**4'-O-methylpyridoxine (MPN).** The toxin 4'-O-methylpyridoxine was not commercially available at the time of the study, so that we synthesized it ourselves according to the method described by Harris (1940).<sup>19</sup> A mixture of 2-methyl-3-hydroxy-4,5-dihydroxymethylpyridine hydrochloride (10.25 g, 50.0 mmol) and sodium methoxide (2.71 g, 50.0 mmol) in methanol (80 mL) was heated in a bomb tube at 130 °C for 12 h. The solvent was evaporated off and the residue treated with acetone and filtered to remove the sodium chloride. The organic solution was concentrated to give an oily residue, which was chromatographed on silica gel. Elution with ethyl acetate-methanol (80:20, v/v) yielded 5.2 g (56.5%) of 2-methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine as an orange oil. The compound was dissolved in ethanol (20 mL) and 2.6 M ethanolic HCl (15 mL) was added while stirring. The suspension was heated at reflux and, after cooling to

0 °C, filtered to give 4.1 g (37.5%) of the compound as a white solid; the final product was chemically characterized to confirm the identity, as described in Results and discussion.

**Ginkgolides.** Reference standards for (–)-bilobalide and ginkgolides A, B, C were kindly supplied by PhytoLab (Vestenbergsgreuth, Germany).

**Reagents.** The reagents used in the study were: acetone for HPLC (Farmitalia Carlo Erba, Milano, Italia); acetonitrile, methanol, water for HPLC and 37% hydrochloric acid (VWR International, Fontenay-sous-Bois, France); absolute ethanol for analysis (Merck KGaA, Darmstadt, Germany); ethyl acetate for HPLC 99.9%, L-ascorbic acid, formic acid ≥98%, and heptafluorobutyric acid ≥99% (Sigma-Aldrich, Steinheim, Germany); ethylenediaminetetraacetic acid (EDTA, Bio-Rad Laboratories, Hercules, California, USA).

## Methods

### Quantification of 4'-O-methylpyridoxine (MPN) in serum

**Preparation of standard solutions and serum sample.** Aliquots of 1 mg of MPN were solubilised in 10 mL of 0.1 M HCl, the final concentration being 100 µg mL<sup>-1</sup>. Further standards of 1 and 0.1 µg mL<sup>-1</sup> (prepared by dilution in 0.1 M HCl) were added to a healthy volunteer's serum present in our seroteque ("blank" sample) to obtain the final concentrations of 4.5, 7.2, 13.5, and 27 ng mL<sup>-1</sup> of MPN, which were used to prepare the test for linear regression. Aliquots of 500 µL of these solutions or of the blank/patient's serum were added to 500 µL of acetone; the resulting solutions were thoroughly vortexed, and centrifuged at 2500 rpm for 5 minutes (Hermle Labortechnik GmbH, Wehingen, Germany). The supernatants were filtered through a 0.45 µm syringe filter (VWR International, Fontenay-sous-Bois, France) and injected into the HPLC.

**Chromatographic conditions.** A HPLC method combined with fluorimetric detection was used for the rapid separation and quantification of 4'-O-methylpyridoxine (MPN) in the patient's serum. The equipment consisted of an Intelligent PU-880 pump (Jasco, Tokyo, Japan), a fluorescence Detector FP-1520 (Jasco, Tokyo, Japan), a sample injection valve Rheodyne 7725 with 20 µL loop (Cotati, California, USA), a column of Inertsil ODS-2, 125 × 4.6 mm, particle size 5 µm (GL Sciences Inc., Tokyo, Japan). ChromNAV software (Jasco, Tokyo, Japan) was used for integration. The analysis was performed by isocratic elution at a flow rate of 1 mL min<sup>-1</sup> with the mobile phase: 0.1% heptafluorobutyric acid in water, and acetonitrile, 90 : 10 (v/v). The column was heated at 40 °C, and the fluorescence detector was set at 290/400 nm ( $\lambda_{\text{ex}}/\lambda_{\text{em}}$ ).

**Method validation.** The HPLC method has been validated as described in Results. The suitability of the chromatographic system was daily checked by using the ChromNAV software (Jasco, Tokyo, Japan).

### Detection of ginkgolides and bilobalide in urine

**Preparation of standard solutions.** Aliquots of 1 mg of each standard (ginkgolides A, B, C and bilobalide) were dissolved in

10 mL of water to a final concentration of 100 µg mL<sup>-1</sup>. Dilutions were carried out in order to obtain a final mixture containing 10 µg mL<sup>-1</sup> of each standard.

**Sample preparation.** The urine sample was prepared according to Dew *et al.* (2014).<sup>20</sup> It was thawed at room temperature immediately before extraction, placed in an ultrasonic water bath (5 min), then vortexed (30 s) to ensure homogeneity. The sample was extracted in triplicate. Each 200 µL aliquot of urine was combined with 20 µL of an aqueous mixture of ascorbic acid (1% w/v) and EDTA (0.1% w/v), 100 µL of ethanol and 1 mL of ethyl acetate in a 1.5 mL centrifuge tube. The solution was vortexed (30 s), placed in an ultrasonic water bath (5 min) and vortexed again to extract soluble compounds. The tubes were centrifuged (Hermle Labortechnik GmbH, Wehingen, Germany) at 17 000 rpm for 10 min at 8 °C and the upper layer was removed. A second 1 mL aliquot of ethyl acetate was added to the pellet, and the extraction procedure repeated. All extracts were dried under nitrogen at room temperature and frozen at -20 °C. On the day of analysis, the second extracts were solubilized in 70 µL of ethanol, vortexed (30 s), placed in an ultrasonic water bath (5 min) and vortexed again. A 50 µL aliquot of this solution was combined with the corresponding first dried extract, which was similarly vortexed and sonicated. A 50 µL aliquot of ascorbic acid solution (0.2%, w/v) was added to the combined extract, which was then vortexed and centrifuged at 17 000 rpm at 8 °C for 10 min. The supernatant was placed in a HPLC vial, which was capped and kept at 8 °C until the analysis.

**Chromatographic conditions.** A HPLC-MS method was applied for the rapid separation of ginkgolides and bilobalide in urine. The equipment included a HPLC Surveyor MS Pump Plus coupled to an ion trap mass spectrometer LCQ Deca XP MAX (Thermo Electron Co, San Jose, CA, USA), a column Kinetex 2.6 µm PFP, 100 Å, 100 × 2.1 mm (Phenomenex, Torrance, California, USA), and a Surveyor Autosampler Plus (Thermo Electron Co, San Jose, CA, USA). The software Excalibur® Release 2.0 SR2 (Thermo Electron, San Jose, CA, USA) was used for integration. The analysis was performed according to Dew *et al.* (2014),<sup>20</sup> using a gradient elution at a flow rate of 0.3 mL min<sup>-1</sup>; the mobile phases were: (A) water-methanol-formic acid, 94.9 : 5 : 0.1 (v/v/v), and (B) methanol-water-formic acid 95 : 4.9 : 0.1 (v/v/v). The gradient elution was set up as shown in Table 1. The autosampler and column oven temperatures were set at 8 °C and 35 °C, respectively, and the

**Table 1** Elution gradient used for the analysis of ginkgolides and bilobalide in urine

Time (min)	Phase A (%)	Phase B (%)
0	92	8
5	92	8
20	60	40
22	10	90
24	10	90
26	92	8
34	92	8

injection volume was 20  $\mu\text{L}$ . MS analysis was performed in negative ionization mode. The capillary temperature was 300  $^{\circ}\text{C}$ , the nebulized gas was nitrogen, sheath gas flow was 50 arb and sweep gas flow was 10 arb.

## Results and discussion

Consumption of *Ginkgo biloba* seeds is quite common in Asian cuisine and in Oriental countries with a normally rather low risk. Traditionally, the seeds are roasted or boiled to reduce the activity of the neurotoxin, 4'-O-methylpyridoxine. Because of the rapid diffusion of oriental cuisine in other countries, the risk could be greater there from the unsuitable intake of ginkgo seeds. Both authorities and the population should be carefully informed of this possibility. To our knowledge, the case of ginkgo seed poisoning described in this paper is the first to occur in Europe.

The objectives of this work were: (1) to confirm the suspected intoxication by *Ginkgo biloba* seeds; (2) to develop accurate methods of measuring biomarkers useful in identifying similar events in the future. In fact, because of the severity of symptoms and the prevalent involvement of children, a clear methodological approach is necessary to allow rapid diagnosis and treatment.

In this case, a 23-months-old male child was brought to the emergency department after tonic-clonic seizures in both arms, and after discussion with the parents we hypothesized that the cause was intoxication by *Ginkgo biloba* seeds, so that serum and urine were collected and immediately sent for biomarker analysis.

On the basis of the paper by Hori *et al.* (2004),<sup>10</sup> a HPLC method for detection of the neurotoxin MPN was set up, with some modifications as described in Methods. Because purified MPN was not available, we synthesized it, as described in Materials and methods. The final product was checked for quality and purity. Purity of 4'-O-methyl pyridoxine was 90%, the remaining 10% being the pyridoxine used as a starting reagent in the synthetic procedure. Since pyridoxine did not interfere with the analysis, this standard was used as a reference compound for quantification, and a correction factor for purity was applied.

The identity of synthesized MPN was confirmed by its melting point of 179–180  $^{\circ}\text{C}$ , and NMR analysis  $^1\text{H}$  NMR (DMSO- $d_6$ )  $\delta$  10.90 (bs, 1 H), 8.13 (s, 1 H), 5.75 (bs, 2 H), 4.68 (s, 2 H), 4.65 (s, 2 H), 3.29 (s, 3 H), 2.63 (s, 3 H). Anal. Calcd for  $\text{C}_9\text{H}_{14}\text{ClNO}_3$ .

A problem arose during the setting up of the method was the different performance shown by MPN standard in water/buffer solutions, compared to human serum or urine. For this reason the linear regressions were prepared in human serum/urine.

### Validation of the method used for the quantification of 4'-O-methylpyridoxine (MPN) in serum

The method for the quantification of MPN in serum/urine was validated according to the guidelines described by Peters *et al.*

(2007),<sup>21</sup> for the following parameters: selectivity, linearity, accuracy, precision and sensitivity (LOD and LOQ). Furthermore, the system suitability test (SST) was performed according to the current FDA Guidelines on Bioanalytical Method Validation.<sup>22</sup> Since MPN was undetectable in child's urine, the relative validation of method is not reported here.

### System suitability test (SST)

The following parameters were calculated: retention factor (K), separation factor between two neighbouring peaks ( $\alpha$ ), peak tailing factor and column efficiency (number of theoretical plates). The analysis was performed in triplicate.

Table 2 shows the results of the SST obtained from the analysis of the "blank" serum spiked with MPN at the final concentration of 30  $\text{ng mL}^{-1}$ .

The parameters show that the chromatographic system used in this assay was highly efficient and suitable for the quantification of MPN, having retention factor (K)  $\geq 2$ , separation factor ( $\alpha$ )  $> 1$ , symmetry factor (SF)  $\leq 2$ .<sup>22</sup>

### Selectivity

The possible interaction between analytes and endogenous matrix components was investigated by adding standard MPN to the "blank" serum (final 30  $\text{ng mL}^{-1}$ ). Peak areas of compounds coeluting with the analytes should be less than 20% of the peak area of sample at LOQ. As shown in Fig. 2, MPN can be clearly identified; no peak at the retention times of MPN had an area exceeding 20% at LOQ values.

### Linearity

Standard solutions (1 and 0.1  $\mu\text{g mL}^{-1}$ ) were added to a "blank" serum to have final concentration of 5, 8, 15 and 30  $\text{ng mL}^{-1}$ ; these solutions were prepared as described in "sample preparation" and analysed with three independent injections.

Linear regression was obtained by plotting the areas of analyte peaks vs. the nominal concentrations. The calibration curve equation and the corresponding correlation coefficient ( $R^2$ ) were calculated taking into account the purity of the standard (90%). The linear regression, used to calculate MPN concentrations in serum is illustrated in Fig. 3. The method was

**Table 2** System suitability test for MPN ( $n = 3$ )

$t_R^a$ (min) (mean $\pm$ SD)	$K^b$ (mean $\pm$ SD)	$\alpha^c$ (mean $\pm$ SD)	SF <sup>d</sup> (mean $\pm$ SD)	$N^e$ (mean $\pm$ SD)
9.76 $\pm$ 0.07	2.86 $\pm$ 0.08	1.21 $\pm$ 0.01	1.01 $\pm$ 0.07	1701 $\pm$ 292

<sup>a</sup>  $t_R$  = Retention time. <sup>b</sup> Retention factor ( $K$ ) =  $(t_R - t_0)/t_0$ , where  $t_R$  and  $t_0$  are retention times of MPN and solvent, respectively. <sup>c</sup> Separation factor ( $\alpha$ ) =  $(t_R - t_0)/(t_{R1} - t_0)$ , where  $t_R$  and  $t_{R1}$  are retention times of MPN and a neighboring peak, respectively. <sup>d</sup> SF (symmetry factor) =  $W_{0.05}/2f$  where  $W_{0.05}$  is width of the peak at 5% height and  $f$  the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline. <sup>e</sup>  $N$  (number of theoretical plates) =  $16/(t_R/W)^2$ , where  $W$  is the peak width at its base.



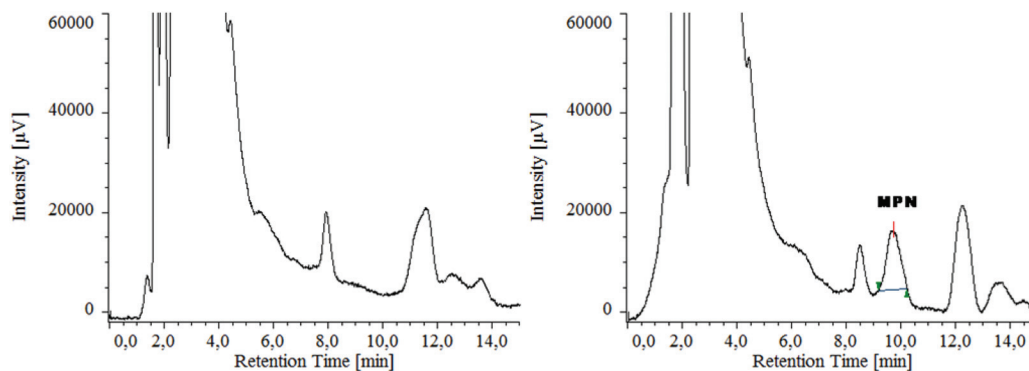


Fig. 2 Chromatograms of a "blank" serum (left panel) and of a "blank" serum added with a standard solution of 4'-O-methylpyridoxine (MPN) at the final concentration of 30 ng mL<sup>-1</sup> (right panel).

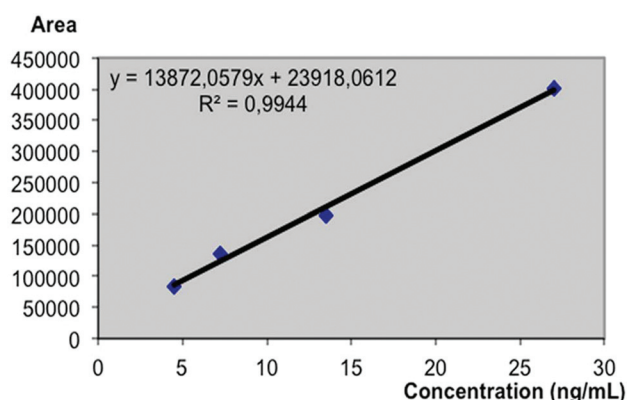


Fig. 3 Linear regression in human serum for MPN quantification.

linear between 4.5 and 27 ng mL<sup>-1</sup>, as shown by the correlation coefficient ( $R^2$ ) higher than 0.99. The linear regression equation was  $y = 13\,872x + 23\,918$ , where  $y$  refers to the peak area and  $x$  refers to concentration.

### Accuracy and precision

Accuracy and precision were determined spiking "blank" serum samples with two concentrations of the MPN standard (0.1 and 1 µg mL<sup>-1</sup>) in order to have final concentrations at 6 and 25 ng mL<sup>-1</sup> (corresponding to actual concentrations of MPN of 5.4 and 22.5 ng mL<sup>-1</sup>, respectively).

Accuracy was calculated as the percent of variation between the experimentally measured means and the nominal values. Precision was determined by calculating the variation coefficient (RSD%) of the peak areas of five replicates injected in the same day.

Table 3 shows the results obtained for accuracy and precision. Being RSD% <15%, the method must be considered precise, and since the calculated accuracy was always within ±15% of the nominal concentration, the method can be considered accurate.

Table 3 Accuracy and precision data ( $n = 5$ )

Standard concentration (ng mL <sup>-1</sup> )	MPN concentration <sup>a</sup> (ng mL <sup>-1</sup> )	Accuracy <sup>b</sup>	Precision <sup>c</sup>
6	5.4	+6.20%	7.36%
25	22.5	+0.07%	7.10%

<sup>a</sup> Allowing for the 95% purity. <sup>b</sup> (Mean of calculated values/nominal value) × 100. <sup>c</sup> Expressed as relative standard deviation (RSD) of calculated values (%).

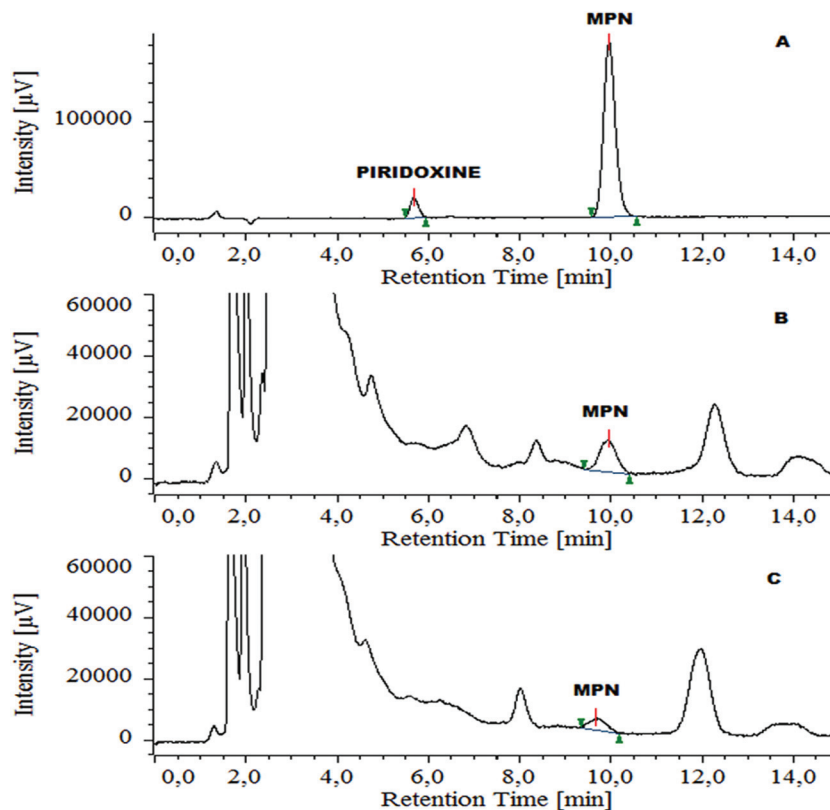
### Sensitivity (LOD and LOQ)

The sensitivity of the analytical method was evaluated by determining the limit of detection (LOD) and the limit of quantitation (LOQ). LOD and LOQ were determined spiking "blank" serum samples with decreasing concentrations of MPN standard: LOD was established at a signal-to-noise ratio of 3 and LOQ at a signal-to-noise ratio of 10. The LOQ was based on data obtained from 5 independent injections.

LOD and LOQ for MPN measured in the "blank" serum were 1.35 and 4.5 ng mL<sup>-1</sup>, respectively. The precision at LOQ was 13.9%, below the acceptable limit of 15%. All values reported above were suitable for an accurate determination of MPN in serum samples.

### Quantification of 4'-O-methylpyridoxine (MPN) in serum

Fig. 4 illustrates the results of MPN quantification in the patient's serum at 14 and 20 hours after eating *Ginkgo biloba* seeds (Panels B and C, respectively). Panel A of the same figure shows the chromatogram of MPN standard solution at the concentration of 0.1 µg mL<sup>-1</sup>. The presence of pyridoxine, as a residue of starting reagent, is clearly evident and was quantified in 10%. As discussed above, the elution time of this "impurity" does not interfere with the MPN determination. MPN concentrations in the child's serum were  $16.53 \pm 1.55$  and  $6.18 \pm 0.69$  ng mL<sup>-1</sup> (m ± SD), at 14 and 20 h from intake, respectively. MPN was also searched for in urine (data



**Fig. 4** HPLC Chromatograms of a standard solution of 4'-O-methylpyridoxine (MPN) at the concentration of  $0.1 \mu\text{g mL}^{-1}$  (A), the patient's serum at 14 h after exposure (B), and at 20 h after exposure (C). All peaks in the chromatogram apart from that of MPN, where present in the blank serum used for developing the method.

not shown) but values were below the limit of detection of  $1.35 \text{ ng mL}^{-1}$ .

#### Identification of ginkgolide terpene lactones in urine

Since MPN was undetectable in urine, a further confirmation of exposure to *Ginkgo biloba* seeds was done by searching ginkgolides and bilobalide in the child's urine. The use of terpenic lactones as urinary biomarkers of ginkgo intake has been considered previously and very recently, interesting results have been published by Dew and coworkers.<sup>20</sup> The chemical structures of these molecules are illustrated in Fig. 1. The detection of ginkgolides (A, B, C) and bilobalide was obtained by HPLC coupled to mass spectrometry. Fig. 5 shows the results obtained by total ion chromatography (TIC) and the single ion monitoring (SIM) for the identification of the *Ginkgo biloba* biomarkers. Panel A shows the chromatograms associated with a mixture of standards at the concentration of  $10 \mu\text{g mL}^{-1}$  for each analyte. The absence of any interfering signal in the "blank" urine is shown in panel B and finally, the presence of ginkgolides in the child's urine is confirmed in panel C (see arrows). The most abundant compound was Ginkgolide B, with a urine concentration of approximately  $40 \mu\text{g mL}^{-1}$ ; Ginkgolide A and C were present in concentrations close to 1 and  $2 \mu\text{g mL}^{-1}$ , respectively. Bilobalide was not detectable.

## Conclusions

The case described in this paper shows that the poisoning due to *Ginkgo biloba* seeds must be considered as a possible clinical event also in European countries, where Asian cuisine has received a relatively recent popularity. Any introduction of new dietetic habits requires suitable information to be provided, as in the case of *Ginkgo biloba* seeds, which are appreciated also by children for their good flavour. Unfortunately, particularly for the youngest children they could represent a risk, as shown by case reports published in the scientific literature.<sup>7,8,10</sup> In the case described here, poisoning was confirmed by identifying biomarkers both in blood and urine. Traditionally, 4'-O-methylpyridoxine (MPN) has been considered as the specific biomarker for ginkgo seed poisoning, both in blood and urine. Considering the different papers reporting cases of ginkgo seed poisoning (Table 4), it quite evident the difficulty in correlating the number of seeds ingested with MPN serum concentration; moreover, the kinetic of MPN in serum and urine after exposure is complex and shows a significant variability.

The case reported here showed a MPN serum concentration of 16.5 and  $6.2 \text{ ng mL}^{-1}$  after 14 and 20 hours from the exposure, respectively. These values are below the concentrations measured by other authors (usually above  $100 \text{ ng mL}^{-1}$ ) but, in agreement with our data, Hasegawa *et al.* (2006)<sup>8</sup>

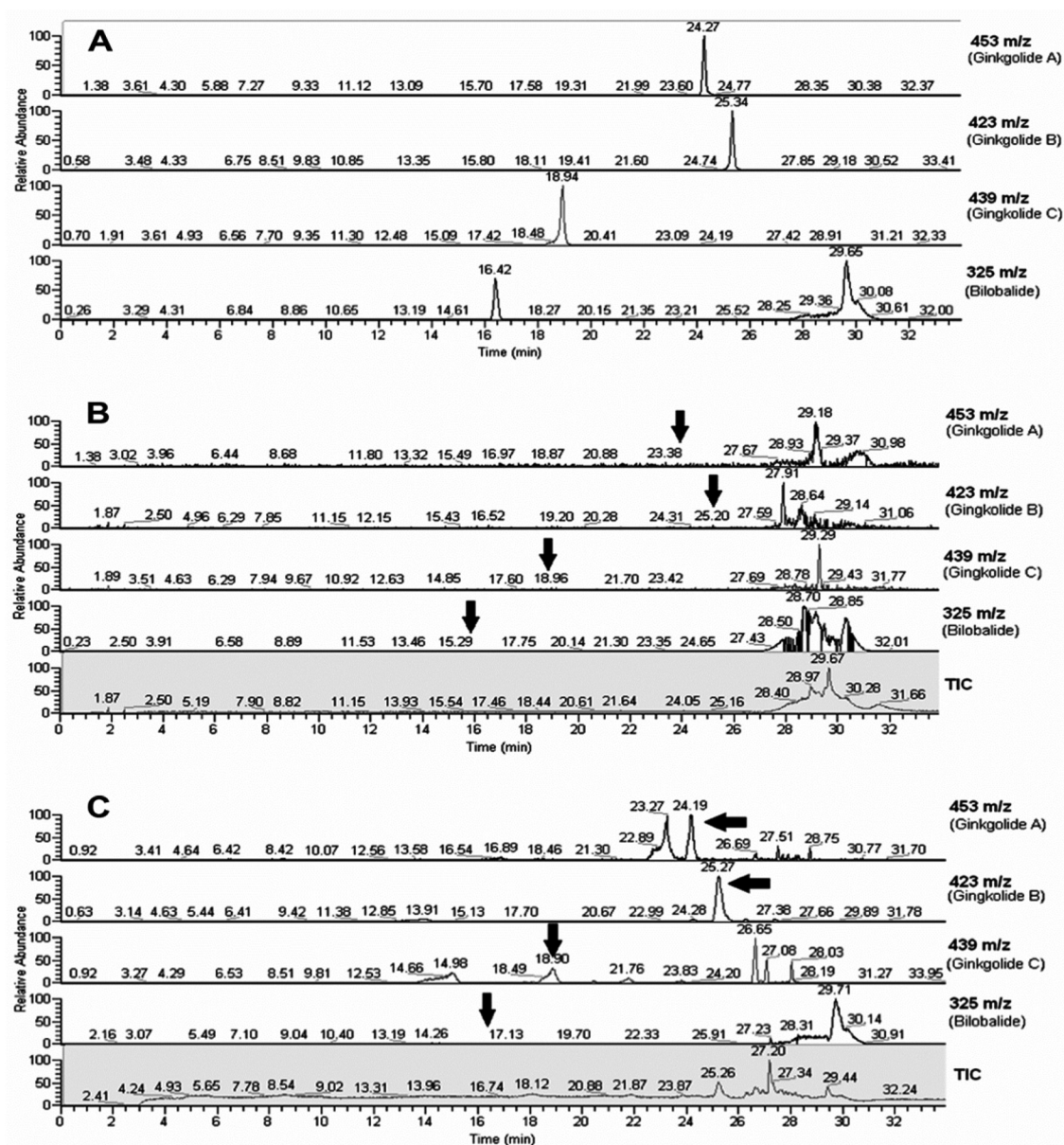


Fig. 5 HPLC/MS of ginkgolide terpene lactones. Panel A = SIM chromatograms of a standard mixture containing ginkgolide A, ginkgolide B, ginkgolide C and bilobalide at the concentration for each analyte of  $10 \mu\text{g mL}^{-1}$ ; panel B = SIM and TIC chromatograms of the "blank" urine; panel C = SIM and TIC chromatograms of the patient's urine (C).

showed that a concentration of  $37 \text{ ng mL}^{-1}$  at 4 hours after the consumption of 50 ginkgo nuts was enough to induce vomiting and afebrile convulsion in a 2-year-old boy. Moreover, Kajiyama & Fjii (2002)<sup>7</sup> showed a very quick decrease of MPN concentration in serum: from  $360 \text{ ng mL}^{-1}$  after 9 hours from the exposure to  $<15 \text{ ng mL}^{-1}$  one hour later, indicating that the timing of sample collection could be critical in detecting the biomarker.

The variability observed in the MPN serum concentration could be justified (at least partially) by the significant difference in MPN content measured in ginkgo seeds ( $172.8\text{--}404.2 \mu\text{g g}^{-1}$  dry weight), supporting also data reported

by different authors where the number of seeds necessary to produce fatalities varied from 15 and 574.<sup>7</sup> According to the case described here, low values of MPN serum concentration can also be responsible for convulsions and adverse effects and it is known that MPN toxicity can be modulated by several factors, such as the patient's nutritional state, where a particular role can be played by the pyridoxine storage.<sup>7</sup>

In contrast to some previous papers, MPN was not detectable in the child's urine, but the ginkgo poisoning was confirmed by the identification of ginkgolide terpenic lactones, which are considered biomarkers for the acute consumption of *Ginkgo biloba* derivatives.<sup>20</sup>



**Table 4** *Ginkgo biloba* seed poisonings: quantification of biomarkers in serum and urine measured by different authors

Reference	Patient age/sex	Seeds consumed	Sample collection (time after exposure)	MPN concentration	
				Serum	Urine
Arahata and Inoue, <sup>24</sup> cited by Hasegawa <i>et al.</i> (2006) <sup>8</sup>	Unknown	Unknown	Unknown	90–494 ng mL <sup>-1</sup>	Not measured
Fujisawa <i>et al.</i> (2002) <sup>25</sup>	17 months/female	10	8 hours	100 ng mL <sup>-1</sup>	Not measured
Hasegawa <i>et al.</i> (2006) <sup>8</sup>	24 months/male	50	4 hours	37 ng mL <sup>-1</sup>	Not measured
			12 hours	57 ng mL <sup>-1</sup>	Not measured
			18 hours	—	397 ng mL <sup>-1</sup>
			38 hours	—	20 ng mL <sup>-1</sup>
Kajiyama & Fjii (2002) <sup>7</sup>	24 months/female	50–60	9 hours	360 ng mL <sup>-1</sup>	Not measured
			10.8 hours	<LOD (15 ng mL <sup>-1</sup> )	
Hori <i>et al.</i> (2004) <sup>10</sup>	24 months/male	50–60	4 hours	1.28 µg mL <sup>-1</sup>	Not measured
	20 months/male	20	8 hours	460 ng mL <sup>-1</sup>	Not measured
	12 months/male	15	3 hours	580 ng mL <sup>-1</sup>	Not measured
			7 hours	420 ng mL <sup>-1</sup>	
	38 years/female	60	12 hours	240 ng mL <sup>-1</sup>	Not measured
	24 months/female	20	5.4 hours	650 ng mL <sup>-1</sup>	Not measured
Ishii <i>et al.</i> (2001) <sup>26</sup> cited by Hori <i>et al.</i> (2004) <sup>10</sup>	Unknown	50	4.5 hours	480 ng mL <sup>-1</sup>	Not measured
Yagi <i>et al.</i> (1993) <sup>27</sup>	21 months/male	50	8.5 hours	90 ng mL <sup>-1</sup>	Not measured
			15.5 hours	<LOD (50 ng mL <sup>-1</sup> )	
This paper	23 months/male	Unknown	14 hours	16.53 ± 1.55 ng mL <sup>-1</sup>	<LOD (1.35 ng mL <sup>-1</sup> )
			20 hours	6.18 ± 0.69 ng mL <sup>-1</sup>	

The correct diagnosis of ginkgo poisoning, is important for suitable treatment of the patient (which usually require vitamin B<sub>6</sub> administration<sup>7,8,10,23</sup>), and to avoid fatalities, so that the identification of MPN in serum or urine could be very useful. A sensitive method for the detection of this biomarker was optimized and validated specifically for the cases of poisoning by ginkgo seeds. We showed in this study that poisoning can be severe also when the level of MPN in urine is not detectable; in these cases, the identification and semi-quantitation of ginkgolide terpene lactones in urine provides a valuable tool in confirming the acute consumption of ginkgo seeds. Moreover, we observed that the ratio between the three main ginkgolides (A:B:C) was approximately 1:40:2. The relative abundance of excreted ginkgolides was different from that observed by Dew *et al.* (2014),<sup>20</sup> where ginkgolide A was the most abundant terpenoid in urine after the consumption of leaf extract (109% of quantity administered). If confirmed by other studies, this specific ginkgolide relative abundance in urine, associated with the absence/very low quantity of bilobalide, could be also used to distinguish between poisoning due to ginkgo leaves and that from seeds. Finally, the case described indicates that population, family doctors, and authorities need to be informed on the specific risk deriving from food containing ginkgo, taking also into consideration the increasing diffusion worldwide of oriental cuisine.

## Conflicts of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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